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Folding kinetics of mammalian ribonucleases

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The folding kinetics of seven different pancreatic ribonucleases are compared both under native conditions and within the unfolding transition. In general, the folding kinetics of these proteins are similar despite numerous amino acid substitutions. Ribonucleases with 4–6 proline residues show 80% slow-folding species. For three ribonucleases with 7 prolines this number increases to 90%. Porcine ribonuclease with a unique Pro 114–Pro 115 sequence folds significantly slower than other ribonucleases which do not show this sequence.

Protein folding *RNase* *Stopped-flow kinetics* *Protein stability* *Proline isomerization*

1. INTRODUCTION

The ordered three-dimensional structure of a protein and the mechanism of its spontaneous formation are both determined by the amino acid sequence of the protein. The 'folding code', by which the sequence dictates folding is still not known. The complex nature of this relationship is illustrated by the finding that proteins with strongly different sequences display very similar three-dimensional structures [1]. Here we ask whether this holds for the acquisition of this structure as well, i.e. whether the mechanism of protein folding is conserved for proteins with similar three-dimensional structures, but with differing sequences. In our experimental approach we compare the kinetics of folding of pancreatic RNases from 7 distinct species. The sequences of more than 40 pancreatic RNases are known to date [2] and the mechanism of folding of the bovine enzyme has been investigated in detail [3–8]. In particular, there is good evidence now that isomerization of Pro 93 is involved in the slow folding of bovine RNase [5–12]. Recently, the folding mechanism of pancreatic RNases which are strongly homologous with the bovine enzyme was found to be highly conserved [13,14]. In this study the folding of RNases is investigated which show variations in sequence of up to 35%. Stopped-flow refolding kinetics are used to measure the fast- and slow-refolding reactions and the slow folding kinetics within the unfolding transition are compared to study the effect of proline substitutions on slow folding.

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Abbreviations: RNase, pancreatic ribonuclease (EC 3.1.27.5); N, native protein; U_F, U_S, fast- and slow-refolding species of unfolded RNases, respectively; GdmCl, guanidinium chloride; τ, time constant of a chemical reaction or phase; α, relative amplitude of a kinetic phase

2. MATERIALS AND METHODS

Bovine RNase A (type XII A) was from Sigma (St. Louis, MO); GdmCl (ultrapure) was from Schwarz-Mann (Orangeburg, NY). Other chemicals were from Merck (Darmstadt, FRG). RNases from sheep (*Ovis aries*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), rat (*Rattus rattus*), cuis (*Galea musteloides*) and pig (*Sus scrofa*) were isolated as described [14–19].

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A Cary 118C spectrophotometer and a Hitachi-Perkin Elmer MPF 44 fluorescence spectrophotometer were used. Refolding experiments were carried out by a 20-fold dilution of unfolded RNase (in 4.0 M GdmCl, 0.1 M glycine, pH 2) with the appropriate refolding solution. Unfolding kinetics were measured after a 10- or 20-fold dilution of native RNase (in H_2O, or in 0.1 M sodium cacodylate, pH 6.0) with the appropriate unfolding solution. Folding was monitored by the absorbance at 287 nm or fluorescence at 305 nm (excitation at 268 nm). Final concentrations of RNase were in the range of 30 μM for absorbance and 10 μM for fluorescence measurements.

Stopped-flow refolding experiments were performed and analyzed as described [13]. Refolding was monitored by absorbance at 287 nm.

3. RESULTS AND DISCUSSION

3.1. *Sequence comparison of the RNases*

In table 1, the individual RNases are compared pairwise both in terms of the absolute number of amino acid replacements and in percent difference of their sequences. RNases from ox and sheep are very closely related, as are the enzymes from the deer species. The RNases from rat, pig and cuis show many differences from the other proteins.

Prolines are assumed to play an important role

Table 1

Similarity of the amino acid sequences of the various RNases[a]

Species→ ↓	Ox	Sheep	Red deer	Roe deer	Pig	Rat	Cuis
Ox	–	4	17	16	28	42	37
Sheep	3%	–	13	12	25	39	35
Red deer	14%	10%	–	5	26	43	39
Roe deer	13%	10%	4%	–	28	43	40
Pig	23%	20%	21%	23%	–	43	33
Rat	34%	31%	35%	35%	35%	–	43
Cuis	30%	28%	31%	32%	27%	35%	–

% amino acid difference

No. of amino acid substitutions

[a] Comparative primary sequence data are found in [2]

Table 2

Distribution of proline residues in the different RNases[a]

	Sequence no.																
	3	4	5	14	15	16	17	18	19	20	21	22	23	24	41	42	43-
Ox															Lys	-Pro	-Val
Sheep															Lys	-Pro	-Val
Red deer				Asp	-Pro	-Ser									Lys	-Pro	-Val
Roe deer				Asp	-Pro	-Ser	-Pro	-Ser							Lys	-Pro	-Val
Pig	Ser	-Pro	-Ala	Asp	-Pro	-Asp									Lys	-Pro	-Val
Rat							Gly	-Pro	-Ser			Ser	-Pro	-Thr	Lys	-Pro	-Val
Cuis								His	-Pro	-Asp					Lys	-Pro	-Val

	Sequence no.														
	-49	50	51	63	64	65	92	83	94	113	114	115	116	117	118
Ox							Tyr	-Pro	-Asn	Asn	-Pro	-Tyr	-Val	-Pro	-Val
Sheep							Tyr	-Pro	-Asn	Asn	-Pro	-Tyr	-Val	-Pro	-Val
Red deer							Tyr	-Pro	-Asn	Asn	-Pro	-Tyr	-Val	-Pro	-Val
Roe deer							Tyr	-Pro	-Asn	Asn	-Pro	-Tyr	-Val	-Pro	-Val
Pig							Tyr	-Pro	-Asn	Asn	-Pro	-Pro	-Val	-Pro	-Val
Rat	Glu	-Pro	-Leu				Tyr	-Pro	-Asn	Asn	-Pro	-Tyr	-Val	-Pro	-Val
Cuis	Glu	-Pro	-Leu	Val	-Pro	-Cys	Tyr	-Pro	-Asn	Thr	-Pro	-Ser	-Val	-Pro	-Val

[a] A recent compilation of sequence data is given in [2]

in slow folding, therefore the proline positions together with their immediate neighbours in the sequence are listed in table 2. The four proline residues (at positions 42, 93, 114 and 117) of the bovine enzyme are conserved in all 7 RNases. One to three additional proline residues occur in the other RNases, most of them in the 15–23 region.

3.2. *Stopped-flow refolding kinetics*

Unfolded bovine RNase A consists of a mixture of fast-refolding (U_F) and slow-refolding (U_S) species, which in refolding give rise to a fast phase in the millisecond time range ($U_F \rightarrow N$, 20%) and a slow phase in the time range of seconds ($U_S \rightarrow N$, 80%) [20]. The refolding kinetics of the various RNases were measured at pH 6 and 35°C after rapid stopped-flow mixing of the unfolded proteins (in 1.4 M GdmCl, pH 1.7) with an equal volume of refolding buffer to give final conditions of 0.7 M GdmCl at pH 6.0. The refolding reactions were monitored by the increase in tyrosine absorbance at 287 nm. The kinetics were analyzed as a sum of exponentials; the time constants and amplitudes for the individual phases are listed in table 3. The results obtained for the RNases from pig, rat and cuis are compared with the refolding data for the proteins from ox, sheep, red deer and roe deer, which were measured previously under the same conditions [13]. These RNases, which contain 4–6 residues, are extremely similar in their refolding kinetics. The distribution of fast- and slow-refolding species, $U_F : U_S$, is invariably 20:80 irrespective of the additional prolines in the deer enzymes. The rates of the corresponding folding reactions are very similar [13,14]. The folding kinetics of the RNases from pig, cuis and rat deviate from this pattern. The amplitudes of the fast-refolding reactions, $U_F \rightarrow N$, are decreased from 20% to about 10%, although the time constants for these reactions remain in the range 70–240 ms. Under the given conditions the slow refolding of the porcine and rat RNases is complex, indicating that the respective U_S species may be heterogeneous. A similar heterogeneity of the slow-refolding reaction (i.e. the presence of two slow-folding species, U_S^I and U_S^{II}) was observed for the other RNases as well, albeit only under conditions which strongly favour the native state [14]. The increase in the fraction of slow-folding species and the more strongly pronounced heterogeneity

Table 3

Refolding kinetics of homologous RNases[a]

Source	No. of prolines	Slow refolding		Fast refolding	
		τ_1 (s)	α_1 (%)	τ_2 (ms)	α_2 (%)
Ox	4	25	80	140	20
Sheep	4	25	79	160	21
Red deer	5	26	81	270	19
Roe deer	6	23	80	160	20
Pig[b]	7	50 1.5	70 20	70	10
Rat[b]	7	30 8.0	68 23	80	9
Cuis[c]	7	46	90	240	10

[a] Unfolding conditions: 1.5 mg/ml RNase in 0.05 M glycine, pH 2, 1.4 M GuHCl, 35°C. Refolding conditions: 0.75 mg/ml RNase in 0.1 M cacodylate, pH 6, 0.7 M GuHCl, 35°C. Refolding was probed by the increase in absorbance at 287 nm

[b] Pig and rat RNase: slow refolding is complex; it was resolved into two phases, I and II, with the time constants τ_1^I, τ_2^{II} and amplitudes α_1^I, α_1^{II}

[c] Slow refolding of cuis RNase is slightly complex; τ_1 is the average rate

of the U_S species of the RNases from pig, rat and cuis may be correlated with the extra proline residues at positions 50 and 64 (rat and cuis) and at 115 (pig). The presence of prolines in the 15–23 region probably does not influence folding. This is supported by the identical refolding kinetics of bovine RNase and the deer RNase (table 3) and by the high frequency of amino acid substitutions found in this chain segment [2], which is exposed to solvent in native bovine RNase [21]. Additional prolines at other positions can lead to an increase in slow-refolding molecules.

3.3. *Slow-folding kinetics in the transition region*

The kinetics of slow folding of the various RNases were measured throughout the GdmCl-induced unfolding transition regions at 10°C and pH 6, the resulting time constants being shown in fig.1. In all cases the folding rates display a minimum within the transition region. The transition midpoints of the various RNases (indicated by arrows in fig.1) are clustered in the region of 3.25–3.60 M GdmCl and the minimum values for the apparent folding rates are at 3.1–3.5 M GdmCl. RNase from cuis is much less stable than the other proteins; its unfolding transition is centered around 2.5 M GdmCl and its rate profile is shifted by about one unit to lower concentrations of GdmCl. For five out of the six RNases that are compared in fig.1 the minimum values for the observed folding rates are remarkably similar; they all lie in a range which corresponds to $\tau = 2900 \pm 300$ s. A strikingly different behaviour is found for porcine RNase. Folding in the transition region is much slower for this protein with $\tau = 6500$ s. In the transition region folding intermediates are not populated [22]. Therefore slow folding should be largely determined by proline isomerization steps. The close correspondence for the rate of slow folding in the transition of the RNases of ox, sheep, red deer, roe deer and cuis indicates that prolines with similar isomerization rates are involved. For bovine RNase Pro 93 is most important for slow folding [5]. Porcine RNase differs from the other RNases not only in its very slow rate of folding in the transition region (fig.1A), but also, after unfolding, the major U_S species of this RNase is formed by an extremely slow process which is absent in other RNases [15]. We suggest that these changes in the folding properties both originate from the presence of the unique Pro 114-Pro 115 sequence in porcine RNase which shows a strongly decreased rate of isomerization.

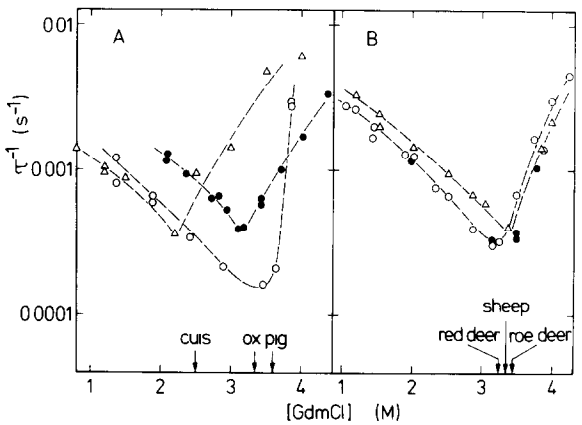

Fig.1. Kinetics of slow folding of various RNases in the GdmCl-induced unfolding transition at pH 6 and 10°C. The dependence on GdmCl concentration of the measured rate constants (τ^{-1}) is shown. Data are derived from both unfolding and refolding experiments. Kinetics were monitored by absorbance at 287 nm and fluorescence at 305 nm. Refolding was carried out by a 20-fold dilution of unfolded RNase (in 4.0 M GdmCl, 0.1 M glycine, pH 2.0) with 0.1 M sodium cacodylate (pH 6.1) and appropriate concentrations of GdmCl. Unfolding was carried out by a 10- or 20-fold dilution of native RNase (in H_2O or in 0.1 M sodium cacodylate, pH 6.0) with appropriate solutions of GdmCl in 0.1 M sodium cacodylate (pH 6.0). (A) Data for RNases from ox (●), pig (○) and cuis (△). (B) Data for red deer (○), roe deer (●) and sheep (△). The arrows indicate the midpoints of the unfolding transitions for the individual RNases. The slow kinetics of rat RNase could not be measured because of the limited amount of material available.

4. CONCLUSIONS

(i) The general folding pattern for different pancreatic RNases is conserved. Fast- and slow-folding reactions are observed throughout.

(ii) An increased number of proline residues leads to an increase in the amount of slow-folding molecules for some RNases. However, RNases with additional prolines in the 15–23 loop only

show unchanged folding properties. This part of the sequence is probably not crucial for folding.

(iii) The rate of slow folding of porcine RNase is decreased compared to all other RNases. This is probably caused by the presence of the unique Pro 114-Pro 115 sequence in this protein.

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